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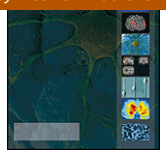

Long-term gender behavioral vulnerability after nociceptive neonatal formalin stimulation in rats

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ABSTRACT

The role of sex and gender in accounting for individual pain behaviors is poorly understood. The present study was conducted to determine whether neonatal nociceptive stimuli at postnatal day 1 (PD1) in rats would lead to a differential behavioral impact based on gender. Animals were divided in 4 groups according to treatment (two injections of 4% formalin into the pad of right paws at PD1 or control) and gender. The sensory threshold and cognition tests were performed in adult rats using the hot plate, open field, elevated plus maze and forced swim tests. The number of paw licks was higher in females and in formalin-treated rats ($P = 0.02$), but without interaction between gender and treatment. Exploratory activity was reduced in males ($P < 0.01$), especially in the nociceptive group ($P < 0.01$). Anxiety levels were higher in the female-nociceptive group ($P < 0.05$). Depression-like behavior was more evident among females, independent of treatment. We concluded that a single acute nociceptive stimulation early in development does not affect nociception and depressive behaviors, but is able to alter the exploratory behavior and anxiety levels in adulthood in a gender specific manner.

Preterm infants are exposed to multiple nociceptive procedures necessary for their survival [7]. Growing evidence from animal [11] and clinical studies [10] have suggested that neonatal nociceptive exposure may result in long-term alterations in sensory processing.

There is a growing interest in the role of gender identity in influencing the prevalence of pain conditions, the response to treatment, or the mechanisms used to cope with challenging pain syndromes. In neonates, the study results are controversial. While some studies showed that female neonates had more pain-associated facial expressions after acute nociceptive stimuli [9] others did not support differences in pain expression between genders [18].

Animal studies that specifically address gender differences after neonatal nociceptive stimulation are scarce [6,11]. These studies take the advantage that at birth, the neurological maturity of neonatal rat pups is similar to that of human preterm neonates at 24 weeks of gestation, reaching full term neonates developmental status around postnatal day 8 (PD8) (for review, see [3]). Considering the significance of preterm neonatal experiences on later cognitive processing, the present study investigated whether nociceptive stimuli triggered by formalin injections in the first postnatal day (PD1) are associated to long-term gender-specific alterations in the sensory processing and behavior of adult rats (PD60–PD67).

Primiparous female Wistar rats with timed pregnancies were obtained from the Animal Services Center (CEDEME) – UNIFESP. The dams were housed individually in plastic cages and maintained on a 12-hour light/dark cycle with free access to food and water. The formalin group consisted of 24 rat pups (12 animals of each gender) that received two unilateral plantar surface injections of 4% formalin (10 μL) into each right paws, with a one hour interval between them, on their first postnatal day (PD1). These groups were named as female-formalin (FF) and male-formalin (MF). Control pups (12 animals of each gender) were handled in the same manner but did not receive any injection and were named as female-control (FC) and male-control (MC). After the formalin injections, dams and pups were kept in the same housing conditions, all breastfed until PD21. During this period no animals presented signs of rejection or negligence. Then they were separated from their mothers and by gender. After that, the animals grew to adulthood undisturbed. During this period, the animals were monitored for abnormal signs indicative of disease (e.g., weight loss, infections, alterations in grooming and other behaviors). All procedures performed in this study were in accordance with and approved by the Animal Care and Use Committee of the Federal University of Sao Paulo (UNIFESP, approval number #2185/08).

Before the behavioral tests, each animal was submitted to a handling procedure. The rats were gently touched and held with two

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gloved hands for approximately five minutes over three consecutive days. All behavioral observations were performed in an isolated room with a temperature of 24 °C. The animals were brought into the room 1 h before training or testing began. All behavioral observations were carried out in the light portion of the light/dark cycle. In the present study, we did not characterize the estrous status of female rats.

On PD60, the rats were placed on 52.5 °C hot plates. Nociceptive thresholds were measured by the latency to the first limb shake or a paw lick and by the number of paw licking. Each animal had a maximal 30 s of exposure time on the hot plate per trial. To avoid potential tissue damage, a 20 s automatic termination of the heat stimulus was imposed if no one withdrawal of the paw did occur. In this case, the animal was scored as having maximal latency (20 s), or no response. Hot plate latency and paw licks were averaged from three trials with 15 min intervals between them. The testing apparatus was thoroughly cleaned between sessions. The observer was blind to treatment condition during testing.

On PD62, the rats were placed in a 40 cm wide, 30 cm deep and 50 cm high wooden box that was painted white during 5 min. Black lines were drawn on the floor to divide the box into 22 quadrants to measure horizontal and vertical exploration as well as crossings and rearing.

On PD64, the animals were tested for anxiety behavior in the elevated plus maze. This consisted of a central platform (5 cm × 5 cm) with two open arms (50 cm long, 10 cm wide and 0.5 cm high borders) and two closed arms (same dimensions the open arms only with 40 cm high walls) that were elevated 50 cm above the ground. Rats were placed on the platform facing the open arm and were observed for 5 min. The total number of entries into the open and closed arms as well as the time spent in the open and closed arms was measured.

The forced swimming test was performed over two days, PD66–PD67. For this, the rats were individually placed in a glass container (height, 50 cm, diameter, 25 cm) filled with warm water (34 ± 1 °C) to a depth of 25 cm. The immobility of the animals (an indicator of depression-related behavior) was timed only in the second day. During the first day, the animals were allowed to know and adapt themselves to water immersion for 15 min. On the second day, the animals were again individually placed in the same water containing glass and the test lasted 5 min. A rat was scored as immobile when it floated passively in the water with more than one third of its tail touching the base of the glass container and it was only making movements to keep its head above the water.

The results are expressed as the mean ± standard error. Comparisons between means were analyzed with a two-way analysis of variance (ANOVA) test using gender and treatment (4% formalin or control) as factors. Differences between the groups were analyzed post-hoc with Tukey's honestly significant difference test (Tukey's HSD). Pearson correlation was used to determine correlations between the tests. The results were considered statistically significant if $P < 0.05$.

Among all studied animals, only one (from the MF group) had signs of motor disability and was excluded from analysis. Control and formalin-stimulated animals from the same gender had similar gain weight curves, reaching similar weight at PD60 (MC = 172.2 ± 10; MF = 167.5 ± 6; FC = 167.2 ± 10; FF = 162.0 ± 7). The following results were obtained from tests conducted between PD60 and PD67.

No significant differences were found in the latency for paw withdrawal among males and females that received or did not receive paw injections of 4% formalin on PD1 (Table 1). However, the number of paw licks was different between both genders (higher in females; $F_{1,41} = 16.7$, $P < 0.0001$) and treatments (higher in formalin-treated animals; $F_{1,41} = 8.6$, $P = 0.005$), but there was no interaction between these variables. The number of licks was higher for the FF group in comparison to FC, and there was statistical significance in the FF versus male-formalin and male-control groups.

The number of rearings (Fig. 1A), crossings (Fig. 1B) and the time spent by the animals in the periphery (1C) of the open field were used as exploratory indexes. The horizontal and vertical exploratory activity was reduced in both male groups compared to females (number of crossings: $F_{1,43} = 277.6$, $P < 0.0001$; number of rearings: $F_{1,43} = 112.3$, $P < 0.0001$). The two-way ANOVA did not detect any interaction between treatment and gender in the number of crossings and rearings; the number of crossings (but not the number of rearings) was reduced in the two formalin-treated groups compared to control groups ($F_{1,43} = 11.9$, $P < 0.001$). Regarding the time spent in the periphery of the open field, the two-way ANOVA identified gender differences (females spent more time in the periphery than males, $F_{1,43} = 46.1$, $P < 0.0001$) and interaction between gender and treatment ($F_{1,43} = 5.3$, $P = 0.02$). Under formalin treatment, however, male rats spent more time in the periphery than the male-control group, while no difference was detected between female groups.

No significant differences were observed in the time spent in the open or closed arms of the elevated plus maze or the number of entries in closed arms among all studied groups of animals (data not shown). The two-way ANOVA identified significant interaction between the number of entries ($F_{1,43} = 13.3$, $P = 0.0007$) and the time spent in the open arms ($F_{1,40} = 6.5$, $P = 0.01$), however, no gender or treatment effects were noted. The female-control group had twice as many entries in the open arm than the female-formalin group ($P < 0.02$), indicating higher anxiety levels for adult females who received a nociceptive stimulus in their early life (Fig. 2). No differences were observed between male groups, besides that males presented opposite responses when compared to female groups.

In the forced swim test, the amount of time that the animal stayed afloat without evident efforts to escape (immobility time – Fig. 3) was used as an index of depression-like behavior. Females showed more depressive-like behaviors than male rats, independent of previous neonatal pain experience ($F_{1,43} = 11.0$, $P = 0.001$). Statistical differences were not detected for treatment and the interaction between pain exposure and gender was absent.

There was a positive correlation between tests related to anxiety levels (time and number of entries in the open arm: $r = 0.6511$, $P < 0.0001$) and tests related to exploratory behavior (number of crossings and rearing in the open field: $r = 0.8206$, $P < 0.0001$; number of crossings and time spent in the periphery in the open field test: $r = 0.5826$, $P < 0.0001$). No significant correlations were detected between the time or number of entries in the open arm

Table 1
Effects of right paw 4% formalin injections at postnatal day 1 on thermal pain threshold.

	Male-control (MM; $n = 12$)	Male-formalin (MF; $n = 11$)	Female-control (FC; $n = 12$)	Female-formalin (FF; $n = 12$)
Latency	12.4 ± 1.7	8.9 ± 0.8	10.5 ± 0.5	10.4 ± 0.5
Lickings	5.9 ± 0.9	7.6 ± 0.8	7.9 ± 0.6*	9.7 ± 0.4*,**

Stimulated rats did not show significant differences in the latency of paw withdrawal when compared to non-stimulated ones from both genders. Female-formalin group had more paw lickings compared to MC (*), $P < 0.001$ and MF (**), $P < 0.05$ groups. Female-control group was significantly different from male-control group (*), $P < 0.05$. The results are expressed as the mean ± SEM (Tukey–Kramer post hoc test).

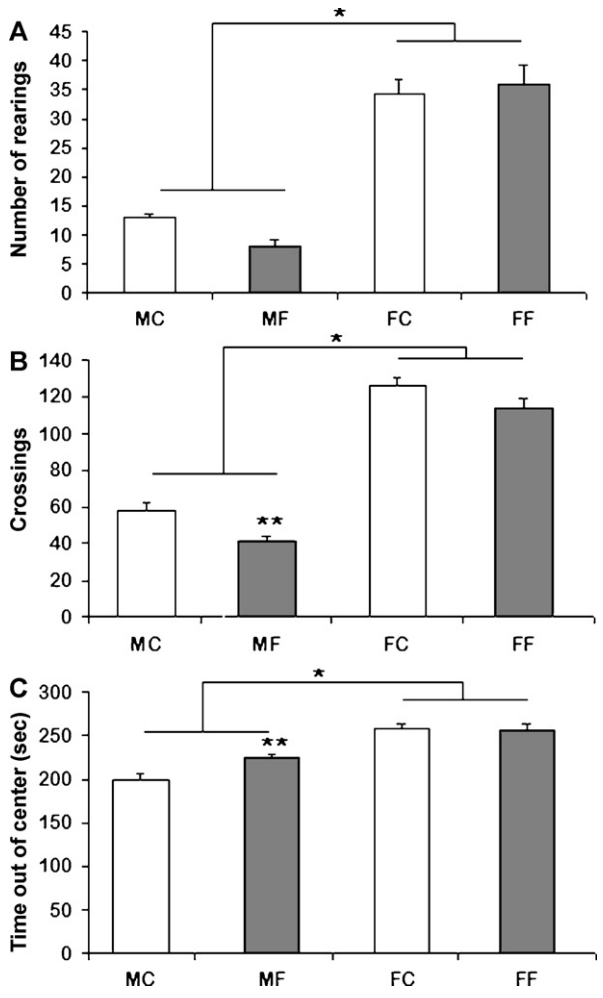

Fig. 1. Effects of intraplantar 4% formalin injection on PD1 on exploratory behavior in the open field test. Male rats displayed less rearing (A) and crossings (B) than females, but did not alter these exploratory parameters in females. Treated males increased the time spent in the periphery (C) compared to the control groups. Results are expressed as the means (±SEM); significance levels: $^{*}P < 0.0001$ for gender, $^{**}P < 0.01$ for treatment. *Abbreviations:* FF, female-formalin; FC, formalin-control; MF, male-formalin; MC, male-control groups.

and the time spent in the periphery of the open field or between the number of licks and other studied parameters.

The present study showed that formalin nociceptive stimuli at postnatal day 1 (PD1) of rat pups lead to long-term specific behavioral effects. Nociceptive stimulation with 4% formalin paw injections at PD1 did not alter the thermal sensitivity and

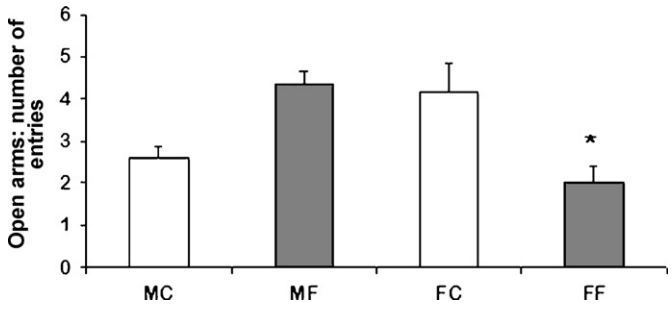

Fig. 2. Effects of intraplantar 4% formalin injections at PD1 on the number open arm entries in the elevated plus maze. The female-nociceptive group displayed less entries ($^{*}P < 0.02$). Female-formalin groups also had less entries in the open arm compared to the male-nociceptive group ($P < 0.05$). Results are expressed as the means (±SEM); abbreviations are the same as used in Fig. 1.

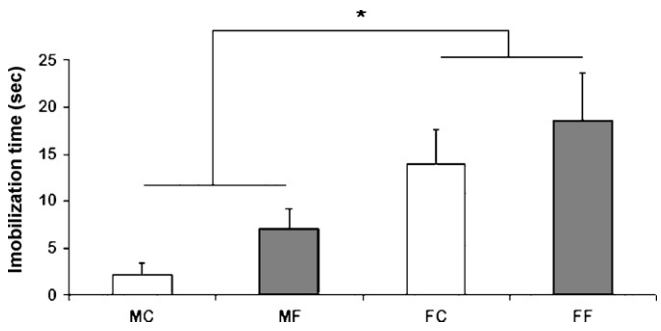

Fig. 3. Effects of intraplantar 4% formalin injections at PD1 on the immobilization time during the forced swim test. Female groups display more depressive-like behavior, despite of neonatal nociceptive stimulation. Results are expressed as the means (±SEM); $^{*}P < 0.01$; abbreviations are the same as used in Fig. 1.

depressive-like behaviors in a gender-dependent way in adults. Adult males were less exploratory than females, a natural feature not altered by the neonatal nociceptive-treatment. However, after formalin-treatment, both genders reduced the crossings and the male adult rats became more protective, spending more time in the periphery of the open field than their controls. After nociceptive neonatal stimuli, adult females but not male rats became more anxious than their controls.

Inevitably, during neonatal intensive care, preterm neonates are subjected to several painful stimuli from routine exposure to diagnostic and therapeutic procedures required for their survival [7]. Injections of formalin [6], complete Freund's adjuvant (CFA) [16] and carrageen [11] have been used to reproduce some of these stimuli in animal models.

Early life nociceptive stimulation may cause permanent changes (for review, see [17]). However, long-term effects of neonatal pain procedures seem to vary according to the experimental paradigm, differences in intensity and duration of the nociceptive stimuli. Daily injections of 10% formalin, from P1 to P7, decreased ethanol preference and reduced locomotor activity in adult rats [6]. Daily needle pricks from P0 to P7 [3] did not produce significant changes in hot plate latency in adulthood. It is interesting to note that despite different concentrations of formalin and/or different number of stimuli, some of the detected behavioral alterations in the previous study [6] were similar to our findings. This suggests that the nociceptive effect of 4% formalin injected at PD1 is sufficient to produce long-term effects on the adult cognition in the animal model.

Regarding possible gender-related differential vulnerability following neonatal nociceptive stimulation, the literature is controversial. Neonatal application of 10% formalin had been shown to produce more effects in males than in female rats, reducing body weight and locomotor activity in males without effect in females [6]. Similarly, 4% formalin injected once a day from P1 to P4 resulted in increased cell death in many cortical brain areas for both genders, reduced exploratory behaviors in adult males, and reduced learning and preparatory behaviors in the radial eight arm maze in females [15]. Here, 4% formalin paw injections (twice in the same day) in males tended to reduce even more their natural low exploratory behavior (when compared to females) besides to shift their preferential central localization in the center of the open field to a more protective position in the periphery. So far, explanations about the physiological mechanisms and brain areas involved these forms of plasticity are poorly known, being mostly related to acute neuronal cell death in specific cortical fields [15].

When nociceptive behaviors are analyzed, more conflicting results can be seen. Either repeated injections of 10% formalin [6] or a single injection of carrageenan on PD1 resulted in significant basal hypoalgesia at adult rats, with more distinct effects in females com-

pared to males [6,11]; while injection of 0.2–0.4% formalin between PD4 and PD14 did not modify adult pain sensitivity [13]. The use of 4% formalin in the current study did not alter paw withdrawals from the heat source but did produce hyperalgesia (increase in paw licks during the hot plate test) among adult female rats. Reduced neuronal activation in the somatosensory cortex has been proposed as a potential explanation for the decreased pain threshold after neonatal nociceptive stimulation [4], but the reasons why males and females respond differently to these distinct intensities or types of neonatal nociceptive stimuli pain require further investigation.

The mechanisms that explain gender specificities in the long-term effects of neonatal nociception were recently reviewed [11]. These specificities, among male and female animals as well as humans, are likely mediated by multiple variables such as the higher expression of neuropeptides in the lumbo-sacral spinal cord of male rats that act as pain inhibitors (met-enkefalin, galanin and cholecystokinin-8) [6]. The significantly higher expression of mu-opioid receptors in the periaquedutal gray area in males [12] provides a mechanism for gender-specific pain processing in the central nervous system. Additionally, the hormonal milieu at birth and during development may influence the different long-term effects of early pain. Males have higher central levels of estradiol at birth compared to females [2,5] and estrogens have been reported to exert neuroprotective effects following acute and chronic injuries in the adult central nervous system [2,8]. Additionally, peripheral injury also results in increased BDNF expression, a factor that is thought to promote neuronal survival and healing [14]. As estradiol increases BDNF expression centrally, this increase may also attenuate the adverse effects of peripheral injury [1]. In this context, a limitation of the present study was the lack of characterization of the estrous status of female rats. The reason for that is related to the gender comparative nature of the current study, where the procedures for such characterization could alter the anxiety levels among females. Thus, future studies might include the monitoring of estradiol levels at adulthood during behavioral testing.

The results found in the present study suggest that the effects of nociceptive stimuli in the neonatal period have sexually dimorphic long-term consequences. The interactions between gender, pain exposure in early life and neurodevelopment need to be explored to better understand the long-term consequences of neonatal intensive care procedures.

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References

[1] A.L. Allen, K.E. McCarson, Estrogen increases nociception-evoked brain-derived neurotrophic factor gene expression in the female rat, Neuroendocrinology 81 (2005) 193–199.
[2] D. Amantea, R. Russo, G. Bagetta, M. Corasaniti, From clinical evidence to molecular mechanisms underlying neuroprotection afforded by estrogens, Pharmacol. Res. 52 (2005) 119–132.
[3] K. Anand, V. Coskun, K. Thrivikraman, C. Nemeroff, P. Plotsky, Long-term behavioral effects of repetitive pain in neonatal rat pups, Physiol. Behav. 66 (1999) 627–637.
[4] K. Anand, W. Sippell, A. Aynsley-Green, Pain, anaesthesia, and babies, Lancet Neurol. 1 (1987) 62–66.
[5] J Balthazart, G. Ball, Is brain estradiol a hormone or a neurotransmitter? Trends Neurosci. 29 (2006) 241–249.
[6] A. Bhutta, C. Rovnaghi, P. Simpson, J. Gossett, F. Scalzo, K. Anand, Interactions of inflammatory pain and morphine in infant rats: long-term behavioral effects, Physiol. Behav. 73 (2001) 51–58.
[7] R. Carbajal, A. Rousset, C. Danan, S. Coquery, P. Nolent, S. Ducrocq, C. Saizou, A. Lapillonne, M. Granier, P. Durand, R. Lenclen, A. Coursol, P. Hubert, L. de Saint Blanquat, P. Boëlle, D. Annequin, P. Cimerman, K. Anand, G. Bréart, Epidemiology and treatment of painful procedures in neonates in intensive care units, JAMA 300 (2008) 60–70.
[8] L. Garcia-Segura, I. Azcoitia, L. DonCarlos, Neuroprotection by estradiol, Prog. Neurobiol. 63 (2001) 29–60.
[9] R. Guinsburg, C. Peres, M. Almeida, R. Balda, R. Berenguel, J. Tonelotto, B. Kopelman, Differences in pain expression between male and female newborn infants, Pain 85 (2000) 127–133.
[10] J. Hohmeister, S. Demirakça, K. Zohsel, H. Flor, C. Hermann, Responses to pain in school-aged children with experience in a neonatal intensive care unit: cognitive aspects and maternal influences, Eur. J. Pain 13 (2009) 94–101.
[11] J. LaPrairie, A. Murphy, Female rats are more vulnerable to the long-term consequences of neonatal inflammatory injury, Pain 132 (2007) S124–S133.
[12] D. Loyd, X. Wang, A. Murphy, Sex differences in micro-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia, J. Neurosci. 28 (2008) 14007–14017.
[13] C. Medeiros, A. Flemeing, C. Johnston, C. Walker, Artificial rearing of rat pups reveals the beneficial effects of mother care on neonatal inflammation and adult sensitivity to pain, Pediatr. Res. (2009) 272–277.
[14] T. Price, M. Louria, D. Candelario-Soto, G. Dussor, N. Jeske, A. Patwardhan, A. Diogenes, A. Trott, K. Hargreaves, C. Flores, Treatment of trigeminal ganglion neurons in vitro with NGF, GDNF or BDNF: effects on neuronal survival, neurochemical properties and TRPV1-mediated neuropeptide secretion, BMC Neurosci. 6 (2005) 4.
[15] C Rovnaghi, S. Garg, R. Hall, A. Bhutta, K. Anand, Ketamine analgesia for inflammatory pain in neonatal rats: a factorial randomized trial examining long-term effects, Behav. Brain Funct. 4 (2008) 35.
[16] M. Ruda, Q. Ling, A. Hohmann, Y. Peng, T. Tachibana, Altered nociceptive neuronal circuits after neonatal peripheral inflammation, Science 289 (2000) 628–631.
[17] W. Sternberg, E. Al-Chaer, Long-term consequences of neonatal and infant pain from animal models, in: K. Anand, B. Stevens, P. McGrath (Eds.), Pain in Neonates and Infants, Elsevier, London, 2007, pp. 57–66.
[18] B. Stevens, C. Johnston, L. Horton, Factors that influence the behavioral pain responses of premature infants, Pain 59 (1994) 101–109.